



Signals in hepatitis A virus P3 region proteins recognized by the ubiquitin-mediated proteolytic system

Vicki P. Losick, Peter E. Schlax, Rebecca A. Emmons, and T. Glen Lawson*

Department of Chemistry, Bates College, Lewiston, ME 04240, USA

Received 19 June 2002; returned to author for revision 7 December 2002; accepted 7 December 2002

Abstract

The hepatitis A virus 3C protease and 3D RNA polymerase are present in low concentrations in infected cells. The 3C protease was previously shown to be rapidly degraded by the ubiquitin/26S proteasome system and we present evidence here that the 3D polymerase is also subject to ubiquitination-mediated proteolysis. Our results show that the sequence $_{32}\text{LGVKDDWLLV}_{41}$ in the 3C protease serves as a protein destruction signal recognized by the ubiquitin-protein ligase E3 α and that the destruction signal for the RNA polymerase does not require the carboxyl-terminal 137 amino acids. Both the viral 3ABCD polyprotein and the 3CD diprotein were also found to be substrates for ubiquitin-mediated proteolysis. Attempts to determine if the 3C protease or the 3D polymerase destruction signals trigger the ubiquitination and degradation of these precursors yielded evidence suggesting, but not unequivocally proving, that the recognition of the 3D polymerase by the ubiquitin system is responsible.

© 2003 Elsevier Science (USA). All rights reserved.

Introduction

In contrast with the rapid infectious cycles of several other picornaviruses, wild-type hepatitis A virus (HAV) infections are characterized by the slow replication of the virus and the typical lack of infection-induced cell lysis (Ticehurst et al., 1989). The reasons for this are not clear, but research to date has uncovered multiple possible explanations (Anderson et al., 1988; Updike et al., 1991; Emerson et al., 1993; Zhang et al., 1995; Yi and Lemon, 2002). Because it is very difficult to detect mature nonstructural HAV proteins in infected cells (Updike et al., 1991), it may be that growth rate is limited, at least in part, by low concentrations of certain viral proteins that can develop during the infectious cycle. Some research has indicated that the slow production of small quantities of progeny HAV can be a consequence of the rate at which the viral RNA is translated (Whetter et al., 1994; Yi et al., 2000), but other data argue that the rate of viral protein synthesis is dependent upon cell type and cannot always be correlated with the

virus replication rate (Schultz et al., 1996; Funkhouser et al., 1999; Graff and Ehrenfeld, 1998). This makes it unlikely that the slow growth of HAV can be attributed exclusively to the slow rate of viral polyprotein production. Because some nonstructural HAV proteins are subject to rapid destruction (Updike et al., 1991; Jia et al., 1991a; Gladding et al., 1997), the rate of HAV replication is also likely to be influenced by selective viral protein degradation.

We previously showed that one HAV nonstructural protein, the 3C protease, is degraded by the ubiquitin/26S proteasome system (Gladding et al., 1997). This system specifically targets proteins for degradation through the assembly of an attached polymeric chain of the 76-amino acid ubiquitin molecule via a hierarchical pathway of three enzymes (Hershko and Ciechanover, 1998; Ciechanover et al., 2000; Pickart, 2001). The pathway begins with the ATP-dependent covalent attachment of ubiquitin to the ubiquitin-activating enzyme, E1. This ubiquitin is then passed on to one of several E2 ubiquitin-conjugating enzymes. Finally, the ubiquitin is transferred to the substrate proteins destined for destruction through the action of E3 ubiquitin-protein ligases. It is the E3 ligases that specifically recognize the substrate proteins, most likely through interactions with structural elements which act as ubiquitination, or protein

* Corresponding author. Fax: +1-207-786-8336.

E-mail address: tlawson@bates.edu (T.G. Lawson).

destruction, signals (Varshavsky, 1996, 1997; Laney and Hochstrasser, 1999; Pickart, 2001). Subsequent iterations of this ubiquitin-conjugating process, operating via an as yet unknown mechanism, result in the attachment of additional ubiquitin molecules, typically with each new ubiquitin being linked to lysine-48 of the previously added ubiquitin (Pickart, 2000). This ubiquitin chain allows the substrate protein to be destroyed by the 26S proteasome complex (Voges et al., 1999; Lam et al., 2002).

Our explorations of the ubiquitin-mediated destruction of the EMCV 3C protease, another rapidly degraded picornavirus processing protease (Lawson et al., 1989, 1994; Oberst et al., 1993), resulted in the discovery that the sequence $_{34}\text{LLVRGRTLTV}_{43}$ in this protein acts as a destruction signal recognized by the ubiquitin-protein ligase E3 α (Lawson et al., 1999). While conducting these studies, we noted that the HAV 3C protease contains a sequence element, $_{32}\text{LGVKDDWLLV}_{41}$, that both shares a weak homology with the mapped EMCV 3C protease protein destruction signal and occurs in an analogous position. Preliminary experiments demonstrated that the replacement of the first three and the last three amino acids in this HAV 3C protease sequence with alanine residues substantially reduced the susceptibility of the protein to conjugation with ubiquitin (Lawson et al., 1999).

Here we report the results of experiments which formally prove that $_{32}\text{LGVKDDWLLV}_{41}$ is in fact a protein destruction signal for the HAV 3C protease. We show that this 10-amino acid sequence is a transferable signal and is required for the efficient conjugation of ubiquitin to the 3C protein. These findings are augmented by our recently published report showing that the ubiquitin-protein ligase E3 α recognizes the HAV 3C protease as a substrate and that mutations in the destruction signal sequence identified here alter the kinetics of E3 α -catalyzed HAV 3C protease ubiquitination (Lawson et al., 2001).

We also evaluated the susceptibility of HAV P3 region polyprotein precursors containing the 3C protease toward ubiquitination. These experiments resulted in the discovery that the HAV 3D RNA polymerase and 3D polymerase-containing polyprotein precursors are subject to ubiquitin-mediated destruction. These findings provide a likely explanation for why the mature viral RNA polymerase is difficult to detect in HAV-infected cells and may contribute to the explanation for why HAV is replicated more slowly than other picornaviruses.

Results

Identification of a protein destruction signal in the HAV 3C protease

To determine if the decameric peptide sequence $_{32}\text{LGVKDDWLLV}_{41}$ in the HAV 3C protease does in fact represent the minimum signal required for the protease to be

targeted for degradation by the ubiquitin-mediated proteolytic system, we tested whether transferring this sequence to another protein converts that protein into a ubiquitin system substrate. While the wild-type poliovirus 3C protease is a very poor substrate for conjugation with ubiquitin, we previously demonstrated that the incorporation of the EMCV 3C protease destruction signal into the poliovirus protein is sufficient to allow both ubiquitination and degradation (Lawson et al., 1999). Using a similar approach, the poliovirus 3C protease sequence $_{28}\text{LGVHDNVAIL}_{37}$, which can be aligned with both the mapped EMCV 3C protease destruction signal sequence and the proposed HAV 3C protease signal sequence (Lawson et al., 1999), was replaced with LGVKDDWLLV. In vitro translation of the RNA transcribed from this plasmid resulted in the synthesis of the poliovirus 3C-DS protease. (DS refers to the proposed HAV 3C protease destruction signal sequence.)

A reaction system containing reticulocyte lysate, the poliovirus 3C-DS protease, and methylated ubiquitin resulted in the synthesis of an easily detected monoubiquitinated conjugate (Fig. 1A, lanes 7 through 9). Typically, 7.0% of the chimeric protein was incorporated into monoubiquitin conjugates, compared with 22% of the wild-type HAV 3C protease (Fig. 1A, lanes 7 through 9 vs lanes 1 through 3, respectively). A much smaller fraction of the wild-type poliovirus 3C protease (less than 2%) was incorporated into ubiquitin-3C protease conjugates in these reaction mixtures (Fig. 1A, lanes 4 through 6). The use of methylated ubiquitin enhances the detection of even low levels of substrate ubiquitination, since polyubiquitin chain synthesis is not supported and the monoubiquitin-substrate conjugate is not efficiently recognized by the 26S proteasome (Hershko and Heller, 1985). Since the reaction system employed included endogenous isopeptidases (Hershko and Ciechanover, 1998), the percentage of substrate protein incorporated into conjugates in this system can only be used for the purpose of comparing the overall extent of ubiquitination that occurred during the incubation period and cannot be taken as a reliable measure of ubiquitination rate.

Additional evidence that the poliovirus 3C-DS protease serves as a substrate for ubiquitination was obtained from experiments in which the protein was incubated in reaction systems supplemented with ubiquitin fused at the N-terminus with glutathione-S-transferase (GST). GST-ubiquitin can replace ubiquitin in ubiquitin-conjugating pathways, and the relatively large size of this fusion protein results in the formation of easily detected high molecular mass polymeric GST-ubiquitin-substrate protein conjugates (Scheffner et al., 1993). As Fig. 1B shows, substantial portions of both the wild type HAV 3C protease and the poliovirus 3C-DS protease were incorporated into easily detected high molecular mass products (17 and 19% in lanes 2 and 6, respectively), while only about 1.6% of the poliovirus 3C protease was conjugated with the polymeric GST-ubiquitin (lane 4).

That the presence of the proposed destruction signal in a

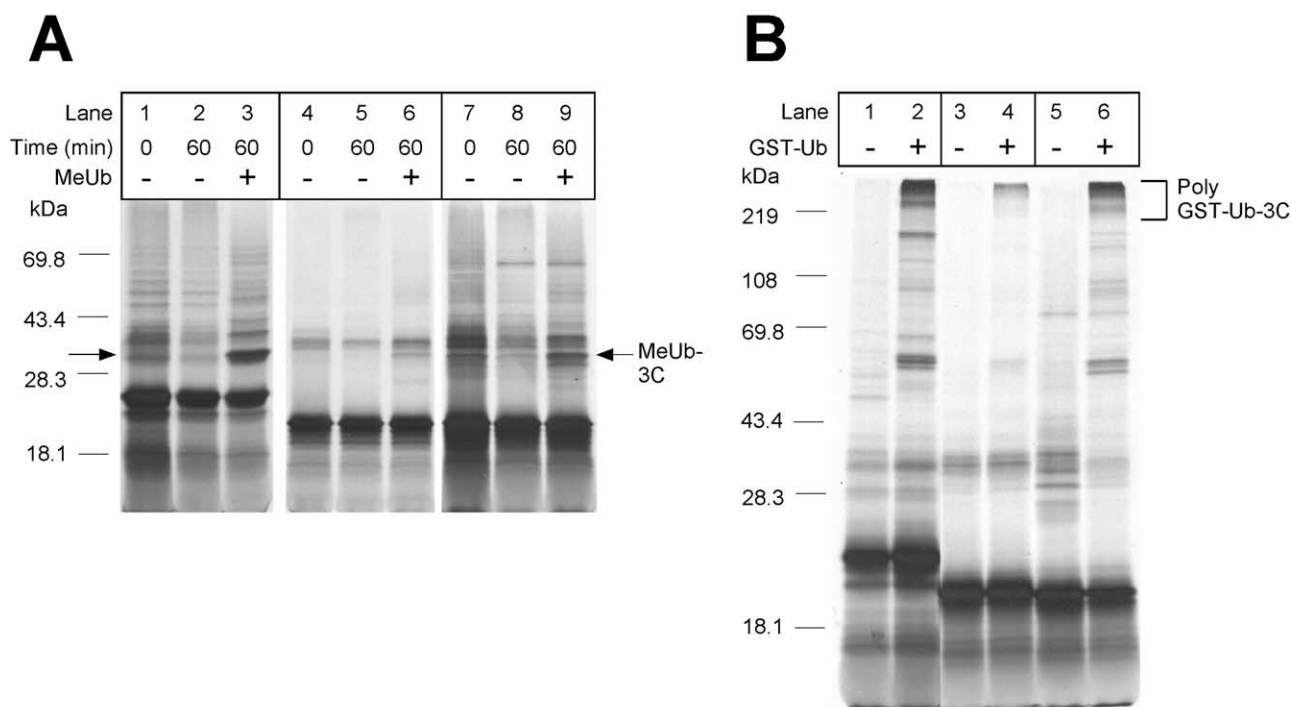


Fig. 1. Evidence that the $^{32}\text{LGVKDDWLLV}^{41}$ sequence in the HAV 3C protease serves as a transferable destruction signal recognized by the ubiquitin-mediated proteolytic system. (A) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which ^{35}S -labeled proteins were incubated for the indicated times in the absence or presence of methylated ubiquitin. Lanes 1 through 3, HAV 3C protease; lanes 4 through 6, poliovirus 3C protease; lanes 7 through 9, poliovirus 3C-DS protease. The arrow indicates the locations of the monoubiquitin-3C protease (MeUb-3C) conjugates. (B) SDS-PAGE analysis of aliquots from reaction mixtures in which the HAV 3C, poliovirus 3C, and poliovirus 3C-DS proteases were synthesized in the presence of GST-ubiquitin. RNA transcripts coding for the 3C proteins were translated in the presence of ^{35}S methionine in reticulocyte lysate in the absence or presence of GST-ubiquitin for 60 min. Lanes 1 and 2, HAV 3C protease; lanes 3 and 4, poliovirus 3C protease; lanes 5 and 6, poliovirus 3C-DS protease. The bracket indicates the location of high molecular mass polymeric GST-ubiquitin-3C protease (Poly GST-Ub-3C) conjugates.

protein triggers degradation is demonstrated by the results of incubating the HAV 3C, the poliovirus 3C, and the poliovirus 3C-DS proteases in mixtures containing reticulocyte lysate and ubiquitin (Figs. 2A and B). Both HAV 3C protease and the poliovirus 3C-DS protease disappear as a function of time, while the wild-type poliovirus 3C protease does not.

Our analysis of the features of the EMCV 3C protease destruction signal suggested that a leucine in the first position of the signal sequence and the presence of a stretch of four, primarily hydrophilic, amino acids between the aliphatic amino acid triplets located at either end of the sequence are required for signal function (Lawson et al., 1999). We tested whether these features are also required in a functional HAV 3C protease protein destruction signal. Two mutated HAV 3C protease proteins were prepared by *in vitro* translation of the RNA encoded for by pHAV3C[+A37] and pHAV3C[L32A]. As the results in Fig. 3A show, both the insertion of an amino acid into the central region of the signal sequence, a change likely to disrupt the higher order structure in the central section of the signal (lanes 7 through 9), and the replacement of the leucine residue in the first position of the signal with an alanine (lanes 10 through 12) sharply reduce the suscep-

tibility of the HAV 3C protease toward conjugation with ubiquitin. Two percent or less of these mutated 3C proteins were incorporated into ubiquitin conjugates. The effect of these mutations is similar to that previously observed for the HAV 3C protease in which the $^{32}\text{LGV}_{34}$ sequence was replaced with AAA (Lawson et al., 1999). The 3C[LGV(32–34)AAA] protein was included in these experiments for the sake of comparison (lanes 4 through 6). That the reduction in susceptibility toward ubiquitination results in the enhanced stability of the mutated 3C proteins is shown by the results in Fig. 3B.

Evaluation of HAV P3 polyprotein precursors and the 3D RNA polymerase as substrates for the ubiquitin/26S proteasome system

To determine if P3 region polyproteins containing the destruction signal in the 3C region are subject to degradation by the ubiquitin/26S proteasome system, we first tested whether the HAV P3 (3ABCD) polyprotein and the 3CD diprotein are substrates for ubiquitination. We originally intended to prepare as substrates for these experiments polyproteins in which the 3C protease active site cysteine codon had been mutated to an alanine codon (C172A). This

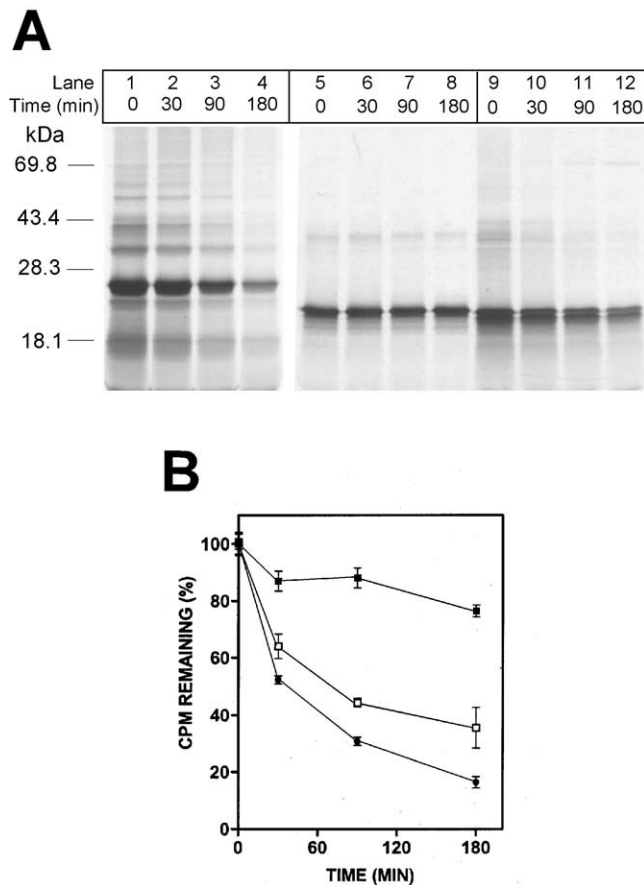


Fig. 2. Comparisons of the stability of the HAV 3C, poliovirus 3C, and poliovirus 3C-DS proteins. ^{35}S -labeled proteins were incubated in reaction mixtures containing reticulocyte lysate and added ubiquitin, and aliquots were removed at the indicated times for analysis by SDS-PAGE. (A) Autoradiograph showing an SDS-PAGE analysis of reaction mixtures containing HAV 3C protease (lanes 1 through 4), poliovirus 3C protease (lanes 5 through 8), and poliovirus 3C-DS protease (lanes 9 through 12). (B) Plots of the ^{35}S -labeled 3C proteins remaining as a function of time. Aliquots were removed from the reaction mixtures for analysis by SDS-PAGE and autoradiography. The amount of radioactivity present in each protein was determined by liquid scintillation counting of gel bands containing the protein and is expressed as a percentage of the amount of radioactivity present at 0 min. The symbols used are as follows: —●—, HAV 3C protease; —■—, poliovirus 3C protease; —□—, poliovirus 3C-DS protease. The bars show the standard deviation values for three averaged measurements.

mutation eliminates the 3C protease-dependent processing of the precursors (Jurgensen et al., 1993). It was determined, however, that this mutation, as well as the more conservative C172S substitution, greatly reduces the ability of the HAV 3C protease alone to serve as a substrate for ubiquitination (data not shown), making these undesirable modifications in the polyproteins. The active site of the folded protein structure (Allaire et al., 1994; Bergmann et al., 1997) is very near the mapped destruction signal sequence, so it is probable that these active site mutations result in higher order structure changes that include the destruction signal region. It was therefore necessary for us to employ

for these studies polyproteins in which the 3C protease catalytic site had not been altered. Since previous studies of HAV polyprotein processing in the reticulocyte lysate system (Jia et al., 1991a; Jurgensen et al., 1993; Tesar et al., 1994) indicated that 3C protease-mediated processing of the P3 region occurs slowly on the time scale of our experiments, we predicted our assay systems would still allow us to detect any significant ubiquitin-precursor conjugate synthesis as well as the degradation of these conjugates.

The HAV 3ABCD polyprotein and the 3CD diprotein were prepared by *in vitro* translation. The masses of the major products from these translation reactions were consistent with those of the HAV 3ABCD and 3CD polyproteins (Fig. 4A, lane 1 and Fig. 4B, lane 1). The majority of the smaller minor products are known to result from aberrant translation initiation events at P3 region AUG codons (Jia et al., 1991b). Incubation of the 3ABCD translation reaction mixtures resulted in the appearance of a small quantity of an approximately 35-kDa product (Fig. 4A, lane 2), previously identified as the 3ABC polyprotein which results from the preferential cleavage of the 3C–3D junction (Jia et al., 1993; Jurgensen et al., 1993; Schultheiss et al., 1994; Tesar et al., 1994). The expected 54-kDa 3D protein product was not obviously apparent, most likely because it comigrates in the SDS-polyacrylamide gels with a minor translation product or because it is degraded in the reaction system (see below). The 3CD diprotein did not appear to undergo significant processing (compare lanes 1 and 2 of Fig. 4B) during the incubation time employed. The incubation of both the HAV 3ABCD polyprotein and the 3CD diprotein in reaction mixtures containing reticulocyte lysate with methylated ubiquitin resulted in the appearance of new discreet higher molecular mass products migrating at positions consistent with monoubiquitinated 3ABCD polyprotein (Fig. 4A, lane 3) and monoubiquitinated 3CD diprotein (Fig. 4B, lane 3), respectively. A relatively small fraction of the polyprotein and diprotein were incorporated into monoubiquitin conjugates in this reaction system (12 and 8.9%, respectively). It is interesting to note that, similar to the HAV and EMCV 3C proteases (Gladding et al., 1997; Lawson et al., 1994), the HAV 3ABCD polyprotein and 3CD diprotein are initially conjugated with ubiquitin at a single site. The inclusion of GST-ubiquitin in the reaction mixtures resulted in the generation of more easily detectable very high molecular mass products with both the 3ABCD polyprotein (Fig. 4A, lane 5) and the 3CD diprotein (Fig. 4B, lane 5). Since it is possible that the polyubiquitination of some of the smaller minor translation products contribute to these high molecular mass conjugates, it is not possible to determine accurately the extent to which the full-length 3ABCD and 3CD polyproteins are incorporated into polymeric GST-ubiquitin conjugates.

To ascertain whether the ubiquitination of the HAV P3 region precursor polyproteins is solely dependent upon the 3C region, we first examined whether the 3D polymerase protein alone is subject to ubiquitination. As shown by the

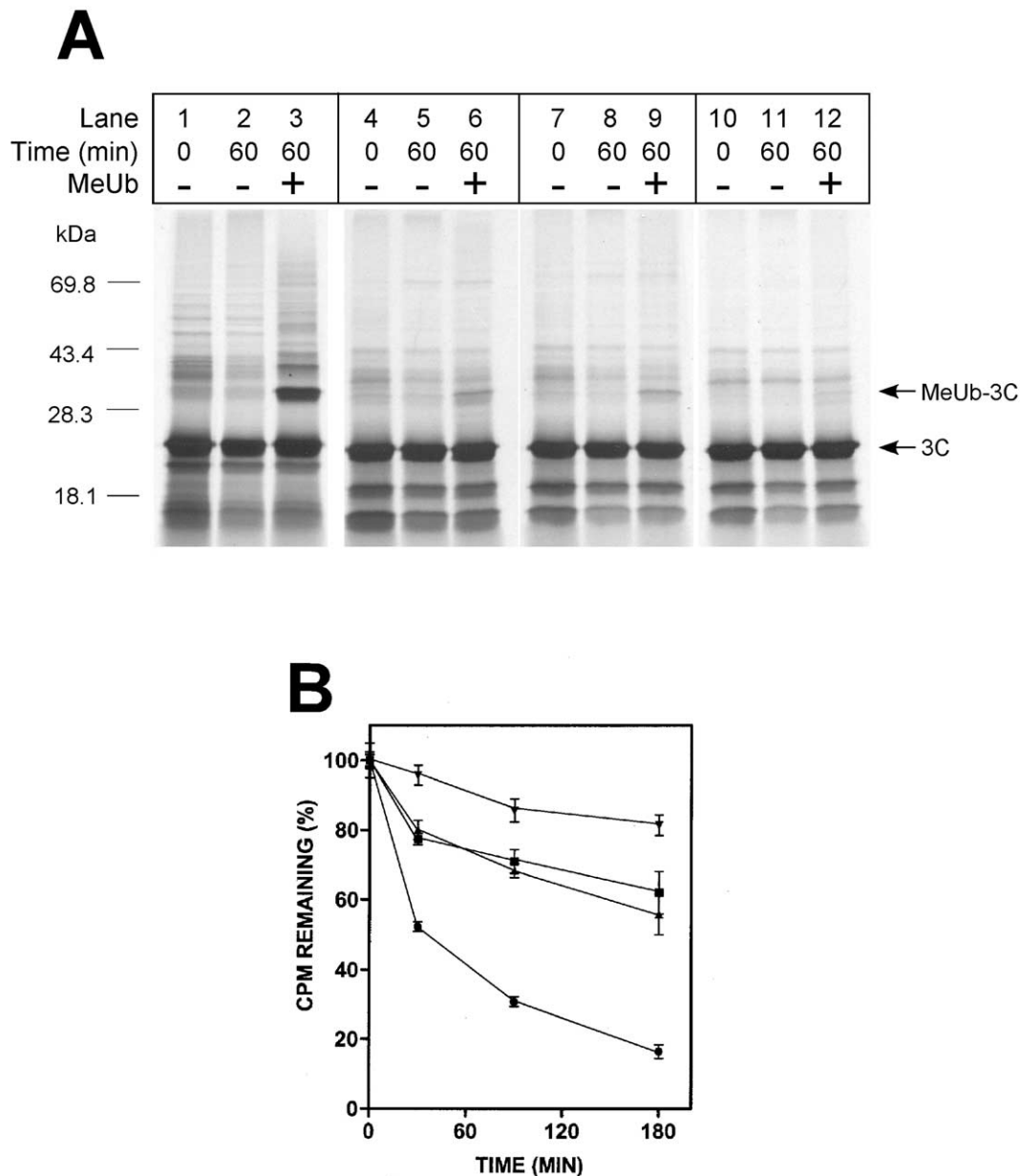


Fig. 3. Evaluation of the susceptibility of HAV 3C protease destruction signal mutants toward ubiquitination and degradation. The sequences of the destruction signal regions of the 3C proteins are as follows: $_{32}$ LGVKDDWLLV $_{41}$, wild-type HAV 3C protease; $_{32}$ AAAKDDWLLV $_{41}$, HAV 3C[LGV(32–34)AAA] protease; $_{32}$ LGVKDDAWLLV $_{41}$, HAV 3C[+A37] protease; $_{32}$ AGVKDDWLLV $_{41}$, HAV 3C[L32A] protease. (A) SDS–PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which 35 S-labeled proteins were incubated for the indicated times in the absence or presence of methylated ubiquitin. Lanes 1 through 3, wild-type HAV 3C protease; lanes 4 through 6, HAV 3C[LGV(32–34)AAA] protease; lanes 7 through 9, HAV 3C[+A37] protease; lanes 10 through 12, HAV 3C[L32A] protease. The arrows indicate the locations of the unconjugated 3C protease proteins and the monoubiquitin-3C protease (MeUb-3C) conjugates. (B) Analysis of the stability of the HAV 3C protease proteins. 35 S-labeled proteins were incubated in reaction mixtures containing reticulocyte lysate and added ubiquitin, and aliquots were removed for analysis by SDS–PAGE and autoradiography. The amount of radioactivity present in each protein was determined by liquid scintillation counting of gel bands containing the protein and is expressed as a percentage of the amount of radioactivity present at 0 min. The symbols used are as follows: —●—, wild-type HAV 3C protease; —■—, HAV 3C[LGV(32–34)AAA] protease; —▲—, HAV 3C[+A37] protease; —▼—, HAV 3C[L32A] protease. The bars show the standard deviation values for three averaged measurements.

results in Fig. 4C, the *in vitro* translated HAV 3D RNA polymerase is ubiquitinated by a conjugating pathway present in rabbit reticulocyte lysate. Incubation of this protein in the presence of either methylated ubiquitin or GST-ubiquitin resulted in the synthesis of the higher molecular

mass products that must be ubiquitin-3D RNA polymerase conjugates. The appearance of a single new product in the presence of methylated ubiquitin indicates that only one ubiquitin is initially attached to the 3D protein; 9.2% of the 3D protein was determined to be present in conjugates with

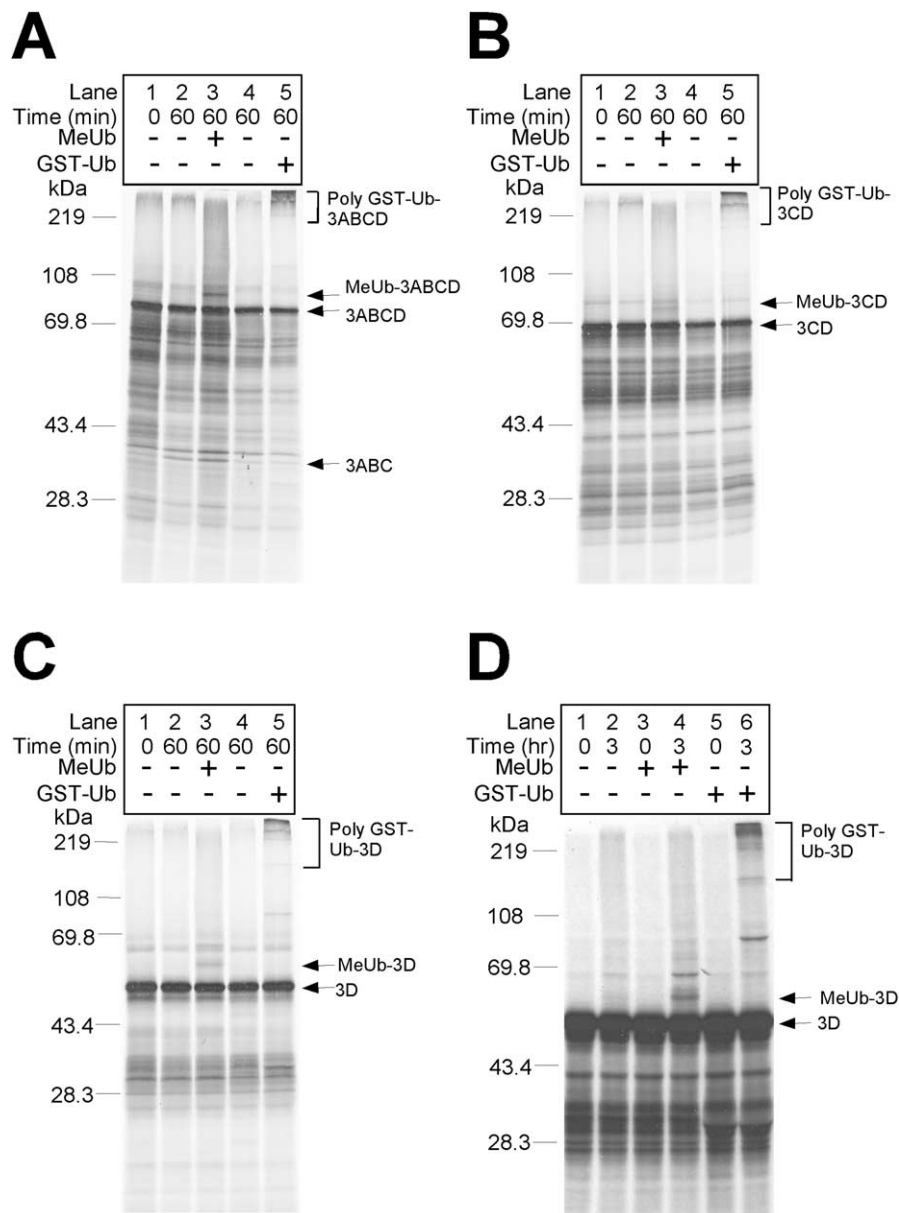


Fig. 4. Evaluation of the HAV 3ABCD polyprotein, 3CD diprotein, and 3D polymerase as substrates for conjugation with ubiquitin. (A) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which ^{35}S -labeled 3ABCD was incubated for the indicated times in the absence or presence of either methylated ubiquitin or GST-ubiquitin. (B) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which ^{35}S -labeled 3CD diprotein was incubated for the indicated times in the absence or in the presence of either methylated ubiquitin or GST-ubiquitin. (C) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which ^{35}S -labeled 3D polymerase was incubated for the indicated times in the absence or in the presence of either methylated ubiquitin or GST-ubiquitin. (D) Results of incubating the HAV 3D polymerase in reaction mixtures containing HeLa cell S-100 extract in the absence or presence of either methylated ubiquitin or GST-ubiquitin. Partially purified ^{35}S -labeled 3D polymerase was incubated for the indicated times in the mixtures, and aliquots were removed for analysis by SDS-PAGE. The arrows indicate the locations of the unconjugated proteins and of the methylated ubiquitin (MeUb) conjugates. The brackets indicate the location of high molecular mass polymeric GST-ubiquitin (Poly GST-Ub) conjugates.

methylated ubiquitin, a value similar to those found for the 3ABCD and 3CD precursors. Given that HAV infects human cells, we tested whether a human ubiquitin-conjugating pathway also recognizes this protein. Partially purified ^{35}S -labeled 3D polymerase was incubated in reaction mixtures containing human HeLa cell S-100 extract, either alone or in the presence of added methylated ubiquitin or GST-ubiquitin. As the results

shown in Fig. 4D indicate, HeLa cells also contain a ubiquitin-conjugating pathway that recognizes the HAV 3D RNA polymerase as a substrate. An important implication of these results is that the 3D polymerase contains a ubiquitin-protein ligase recognition signal which might also play a role in the recognition and ubiquitination of the 3D polymerase-containing precursor polyproteins.

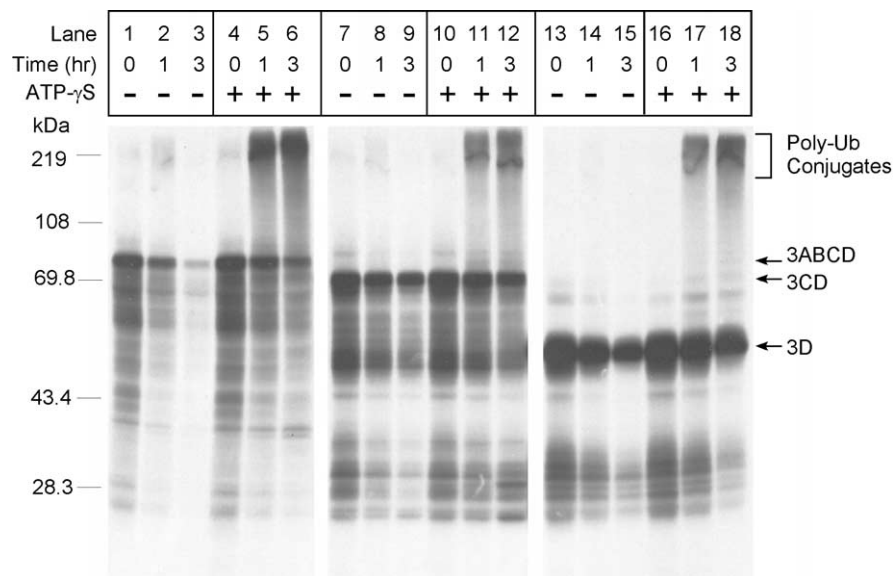


Fig. 5. Effects of ATP- γ -S on the stability of the HAV 3ABCD polyprotein, 3CD diprotein, and 3D polymerase. 35 S-labeled proteins were incubated in reaction mixtures containing reticulocyte lysate and added ubiquitin in the absence or presence of ATP- γ -S, and aliquots were removed at the indicated times for analysis by SDS-PAGE. The arrows indicate the locations of the substrate proteins. The bracket indicates the location of accumulated polyubiquitin (Poly-Ub) conjugates.

We previously observed that the EMCV 3D RNA polymerase generated by the processing of the EMCV 3ABCD polyprotein is not subject to rapid destruction (Lawson et al., 1994). Evaluations of the susceptibility of in vitro synthesized EMCV 3D RNA polymerase, as well as poliovirus 3D polymerase, toward ubiquitination in the reticulocyte lysate reaction system revealed that both of these proteins are poor substrates for ubiquitin attachment (data not shown).

It was important to determine whether the ubiquitinated HAV 3ABCD polyprotein, 3CD diprotein, and 3D RNA polymerase are degraded by the 26S proteasome. The disappearance of all three proteins as a function of time in the reticulocyte lysate-containing reaction system is evident from the results shown in Fig. 5, lanes 1 through 3, 7 through 9, and 13 through 15, with the 3ABCD polyprotein being degraded more rapidly than the other two substrates (see below). The inclusion of adenosine 5'-O-(3-thiotriphosphate) (ATP- γ -S), which supports the synthesis of ubiquitin-protein conjugates but does not support ATP-dependent proteolysis by the proteasome (Ciechanover et al., 1994; Lawson et al., 1994; Gladding et al., 1997), resulted in the accumulation of higher molecular mass products (Fig. 5, lanes 4 through 6, 10 through 12, and 16 through 18) characteristic of those found in heterogeneous mixtures of polyubiquitinated proteins. These results serve to both confirm that the HAV 3ABCD polyprotein, 3CD diprotein, and 3D RNA polymerase are subject to ubiquitination and provide evidence that the polyubiquitinated proteins are substrates for proteolysis by the 26S proteasome.

The presence of a unique *Xho*I site in the pHAV3D plasmid allowed us to test whether removing 137 amino

acids from the carboxyl-terminus of the HAV 3D polymerase also removes all or part of a protein destruction signal. The incubation of this protein in reaction systems containing reticulocyte lysate and methylated ubiquitin or GST-ubiquitin resulted in the appearance of the new higher molecular mass products that indicate this protein is a substrate for ubiquitination (Fig. 6). The portion of the truncated 3D protein determined to be conjugated with methylated ubiquitin (7.8%) is similar to that measured for the full-length 3D polymerase. Features in the HAV 3D polymerase required for recognition by the ubiquitin-conjugating system therefore appear to reside within the amino-terminal 352 amino acids.

The potential for the HAV 3D RNA polymerase ubiquitination signal to contribute to the recognition of the P3 polyprotein precursors by the ubiquitin-conjugating system was more carefully examined. Plasmids containing the HAV 3ABCD and 3CD coding sequences in which the $_{39}\text{LLV}_{41}$ coding sequence in the 3C protease gene was mutated to AAA (Lawson et al., 1999) were constructed. These constructs were designated as pHAV3ABC(i)D and pHAV3C(i)D, with (i) indicating the presence of an inactivated 3C protease destruction signal. In vitro translation of the RNA transcribed from these plasmids resulted in the synthesis of products similar to those obtained from the unmutated RNA transcripts, with the exception that no processing of the mutated 3ABC(i)D polyprotein was detected (compare Fig. 7A, lanes 1 and 2 with Fig. 4A, lanes 1 and 2). The LLV(39–41)AAA mutation in the HAV 3C protease, being near the catalytic site in the folded protein, likely reduces or eliminates the 3C protease activity. Both the mutated 3ABC(i)D polyprotein and the 3C(i)D diprotein

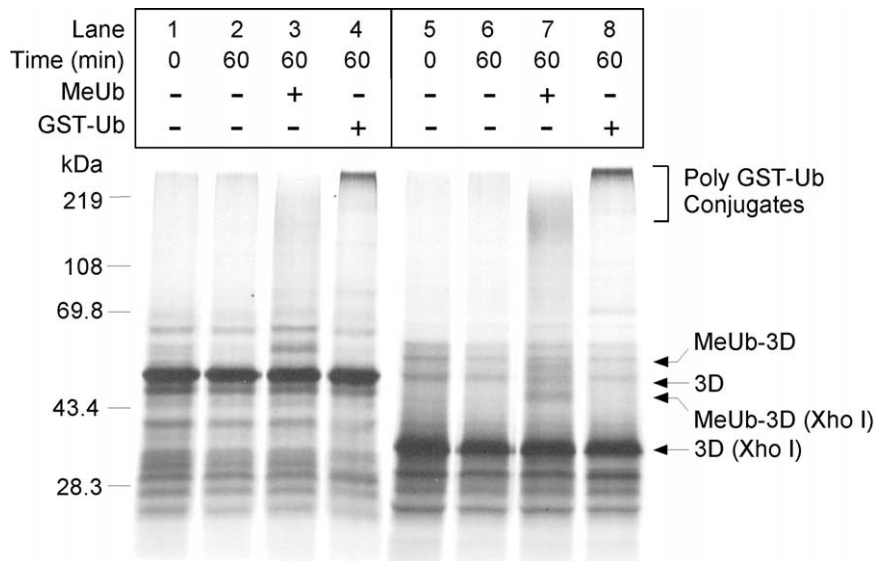


Fig. 6. Evaluation of HAV 3D polymerase lacking 137 carboxyl terminal amino acids as a substrate for ubiquitination. ^{35}S -labeled full-length 3D polymerase (lanes 1 through 4) or the 3D polymerase synthesized from RNA transcribed from pHAV3D digested with *Xho*I (3D (*Xho*I) protein; lanes 5 through 8) was incubated in the absence or presence of either methylated ubiquitin or GST-ubiquitin. Aliquots were removed from the mixtures at the indicated times for analysis by SDS-PAGE. The arrows indicate the locations of the unconjugated proteins and of the methylated ubiquitin (MeUb) conjugates. The bracket indicates the location of high molecular mass polymeric GST-ubiquitin (Poly GST-Ub) conjugates.

were subject to ubiquitination (Figs. 7A and B). The mutation of the mapped destruction signal in the 3C protease region did not appear to affect the susceptibility of the poly- and diproteins toward ubiquitination since, for example,

11% of the 3ABC(i)D polypeptide and 8.9% of the 3C(i)D diprotein were incorporated into conjugates with methylated ubiquitin, percentages similar to those measured for the unmutated 3ABCD polypeptide and 3CD diprotein.

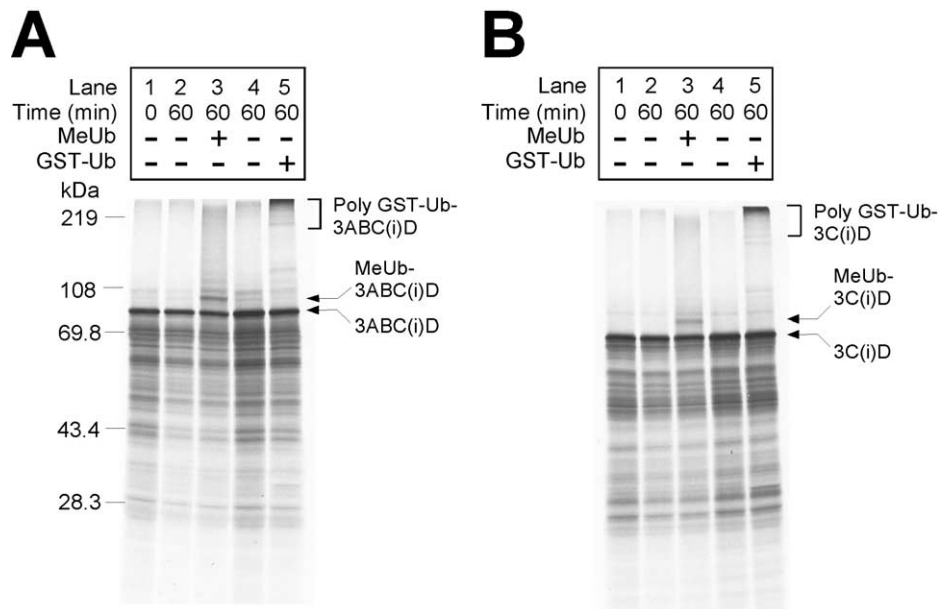


Fig. 7. Evaluation of the effect inactivating the HAV 3C protease destruction signal has on the ubiquitination of 3C protease-containing polyproteins. The wild-type 3C protease destruction signal sequence $_{32}\text{LGVKDDWLLV}_{41}$ in these proteins was mutated to $_{32}\text{LGVKDDWAAA}_{41}$ (termed LLV(39–41)AAA) to generate an inactive (i) signal. (A) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which ^{35}S -labeled 3ABC(i)D polypeptide was incubated for the indicated times in the absence or presence of either methylated ubiquitin or GST-ubiquitin. (B) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which 3C(i)D diprotein was incubated for the indicated times in the absence or presence of either methylated ubiquitin or GST-ubiquitin. The arrows indicate the locations of the unconjugated and the methylated ubiquitin (MeUb) conjugates. The bracket indicates the location of high molecular mass polymeric GST-ubiquitin (Poly GST-Ub) conjugates.

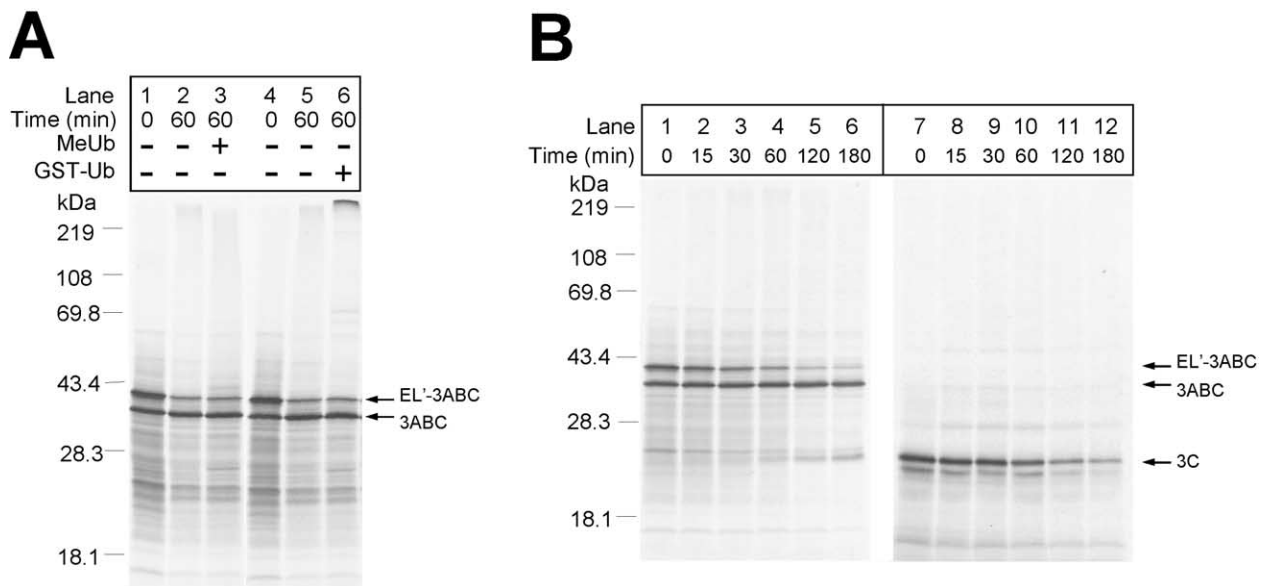


Fig. 8. Evaluation of the HAV 3ABC polyprotein as a substrate for ubiquitination. (A) SDS-PAGE analysis of aliquots from reaction mixtures containing reticulocyte lysate in which ^{35}S -labeled EL'-HAV 3ABC (EL'-3ABC) polyprotein was incubated for the indicated times in the absence or presence of either methylated ubiquitin or GST-ubiquitin. (B) Evaluation of the processing of the EL'-HAV 3ABC polyprotein and the stability of the EL'-HAV 3ABC and 3ABC polyproteins. ^{35}S -labeled EL'-HAV 3ABC polyprotein or HAV 3C protease were incubated in reaction mixtures containing reticulocyte lysate and added ubiquitin, and aliquots were removed at the indicated times for analysis by SDS-PAGE. The arrows indicate the locations of the major products.

Supporting evidence for the involvement of a feature in the 3D RNA polymerase, as opposed to a site in another protein region, in the recognition of HAV P3 polyprotein precursors was obtained by evaluating the susceptibility of the 3ABC polyprotein as a substrate for the ubiquitin/26S proteasome system. Attempts to synthesize the 3ABC polyprotein from RNA transcripts prepared from plasmids carrying the polyprotein coding sequence immediately downstream of a promoter were unsuccessful. To overcome this problem, the unmutated 3ABC polyprotein coding region was inserted into the EMCV leader protein-encoding sequence located downstream of the EMCV 5' untranslated region containing the IRES sequence (Jia et al., 1993). RNA transcribed from the resulting plasmid, pE5L'-HAV3ABC, codes for the HAV 3ABC polyprotein fused with MATT-MEQETCAQ (designated here EL', for truncated EMCV leader) at the N-terminus and RIMKV at the C-terminus. This RNA was found to be translated efficiently in reticulocyte lysate, although the major product appeared to undergo a processing event to generate a new product with a mass consistent with that of the 3ABC polyprotein (Fig. 8A, lanes 1 and 2). Since a Q-G junction is generated by the N-terminal 12 amino acid long fusion polypeptide, the most likely explanation for the observed processing is the 3C protease-dependent cleavage of this junction to release the 1325-Da dodecamer. As shown by the results in Fig. 8A, incubating these translation and processing products in reaction mixtures containing either methylated ubiquitin or GST-ubiquitin resulted in the synthesis of relatively small amounts of either monoubiquitinated 43-kDa 3ABC polyprotein (less than 2% of substrate incorporated; lane 3)

or polyubiquitinated conjugates (less than 5% of substrate incorporated; lane 6). The relative inefficiency of 3ABC polyprotein ubiquitination becomes evident if one compares the fraction of the 3ABC polyprotein incorporated into ubiquitin conjugates with the fraction of the HAV 3C protease which is moved into higher molecular mass material during incubations under the same conditions (Fig. 1A, lane 3 and Fig. 1B, lane 2).

The incubation of the 3ABC fusion protein translation products for longer periods of time in reaction mixtures containing added ubiquitin demonstrates even more clearly the time-dependent removal of the N-terminal 12 amino acid polypeptide and shows that mature 3C protease is released slowly (Fig. 8B, lanes 1 through 6). While it is difficult to quantitatively evaluate the stability of the 3ABC polyprotein in this system, a direct comparison with the stability of the 3C protease alone (Fig. 8B, lanes 7 through 12) demonstrates that the mature 3C protease disappears more quickly than the total population of 3ABC polyprotein-containing products. This is consistent with previously reported results identifying the 3ABC polyprotein as a processing intermediate that tends to accumulate (Jia et al., 1993; Jurgensen et al., 1993; Schultheiss et al., 1994; Probst et al., 1998). These results suggest that the presence of the 3AB region on the N-terminus of the 3C protease, without the presence of the 3D polymerase, is detrimental to the recognition of the polyprotein by the ubiquitin system.

An attempt was made to determine the extent to which targeting of the HAV 3ABCD polyprotein and 3CD diprotein for degradation depends upon the 3D polymerase ubiquitin-conjugating signal vs the mapped signal in the 3C

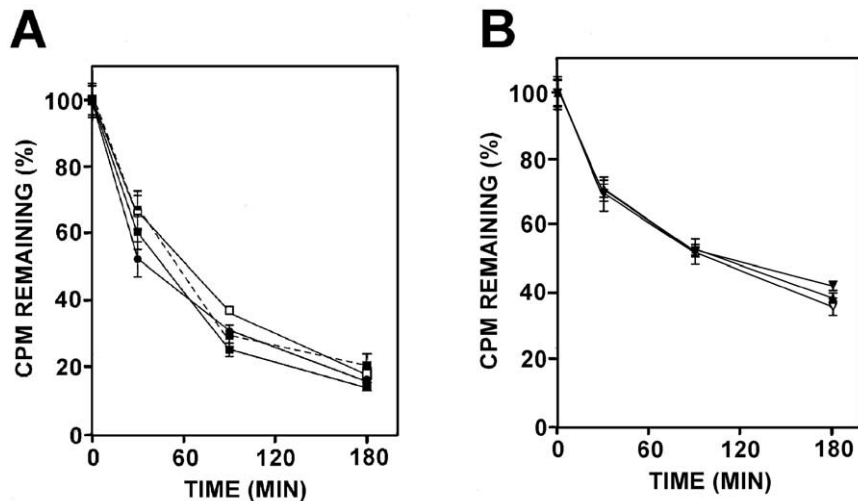


Fig. 9. Evaluation of the effect of a nonfunctional (LLV(39–41)AAA) HAV 3C protease destruction signal (3C(i)) on the degradation rates of the HAV 3ABCD polyprotein and 3CD diprotein. ^{35}S -labeled proteins were incubated in reaction mixtures containing reticulocyte lysate and added ubiquitin, and aliquots were removed for analysis by SDS–PAGE at the indicated times. The amount of radioactivity present in each protein was determined by liquid scintillation counting of gel bands containing the protein and is expressed as a percentage of the amount of radioactivity present at 0 min. The bars show the standard deviation values for three averaged measurements. (A) A comparison of the degradation rates of the 3ABCD polyproteins and the 3C protease. The symbols used are as follows: —■—, 3ABCD polyprotein; —▣—, 3ABCD plus 3ABC polyproteins; —□—, 3ABCD polyprotein containing the mutated destruction signal sequence; —●—, 3C protease. (B) A comparison of the degradation rates of the 3CD diproteins and the 3D polymerase. The symbols used are as follows: —▼—, 3CD diprotein; —▽—, 3CD diprotein containing the mutated destruction signal sequence; —▲—, 3D polymerase.

protease. The kinetics with which the 3ABCD polyprotein and 3CD diprotein, unmodified or containing the LLV(39–41)AAA mutation in the 3C protease destruction signal sequence, are degraded were compared. As Fig. 9A shows, both the unmutated and the mutated 3ABCD polyproteins were rapidly degraded at similar rates, these rates being comparable to that of the degradation of the HAV 3C protease. (Included here are the labeled protein amount vs time measurements for both the unmutated 3ABCD polyprotein only and the 3ABCD polyprotein plus the detectable 3ABC polyprotein that results from processing during the incubations. The inclusion of the small amounts of this product in the measurements does not have a large impact on the calculated polyprotein disappearance rate.) The unmutated and mutated 3CD diprotein, as well as the HAV 3D polymerase, were degraded at virtually identical rates (Fig. 9B). Both versions of the 3CD diprotein and 3D RNA polymerase were degraded more slowly than the 3ABCD polyproteins or the mature 3C protease. While the similar kinetics with which the 3CD diproteins and 3D polymerase are degraded argue that it is a feature of the 3D, and not the 3C, region of the diprotein that is recognized by the ubiquitin-conjugating machinery, the results obtained with the 3ABCD polyprotein are difficult to interpret. Even with the mapped destruction signal in the 3C region inactivated, the polyprotein was degraded with kinetics that more closely resemble those for the 3C protease degradation than for the 3D RNA polymerase destruction. If, as the findings described above suggest, it is a signal in the 3D region that is primarily responsible for the 3ABCD polyprotein serving as a ubiquitin system substrate, then inactivat-

ing the mapped 3C protease destruction signal would be expected to result in degradation kinetics that match those of the 3D polymerase.

Discussion

We have shown here that the sequence $_{32}\text{LGVKD-DWLLV}_{41}$ acts as a protein destruction signal that directs the ubiquitination of the HAV 3C protease. The HAV 3C protease signal maps at a position analogous to that of a similar signal sequence present in the EMCV 3C protease (Lawson et al., 1999) and occurs in a strand-turn-strand motif, with the central region exposed on the surface of the protein (Allaire et al., 1994; Bergmann et al., 1997). Our data demonstrate that the functional HAV 3C protease destruction signal must contain features similar to those found in the EMCV 3C protease signal (Lawson et al., 1999). The results of a recent study of the kinetics of the ubiquitin-protein ligase E3 α -catalyzed ubiquitination of picornavirus 3C proteases revealed that E3 α recognizes the HAV 3C protease as a substrate and that this recognition involves interactions with the $_{32}\text{LGVKDDWLLV}_{41}$ region (Lawson et al., 2001). This indicates that the mapped destruction signal in the HAV 3C protease, similar to that identified in the EMCV 3C protease (Lawson et al., 1999), is recognized by E3 α .

We also discovered that the HAV 3ABCD polyprotein, 3CD diprotein, and the 3D RNA polymerase are all ubiquitinated and subject to proteolysis by the 26S proteasome. These results are significant, because they show that the

accumulation of very small amounts of the 3D polymerase in virus-infected cells (Updike et al., 1991) can be explained, at least in part, by the degradation of the protein and its precursors. It is interesting to note that the 3D RNA polymerases of both EMCV and poliovirus are poor ubiquitin system substrates and that these proteins do accumulate in the host cells (Jen and Thach, 1982; Charini et al., 1991). This correlation between 3D polymerase stability and the duration of the respective virus replication cycles may indicate that RNA polymerase concentration can be an important factor in determining picornavirus growth rates.

The HAV 3D RNA polymerase must be recognized by at least one ubiquitin-protein ligase that most likely interacts with a particular structural motif that acts as a ubiquitination/protein destruction signal. We were able to demonstrate that the destruction signal does not require the carboxyl-terminal 137 amino acids. A preliminary evaluation of a fragment composed of the amino-terminal 97 amino acids of the 3D protein has provided evidence that this region alone is not a substrate for ubiquitination (T.G. Lawson, unpublished results). It should be noted that the mature HAV 3D polymerase has an N-terminal arginine, which is one of the amino acids known to allow proteins to serve as substrates for the E3 α -mediated N-end rule ubiquitination pathway (Reiss et al., 1988; Varshavsky, 1996). The version of the RNA polymerase employed here was synthesized with a stabilizing N-terminal methionine, so it seems unlikely that E3 α is recognizing our substrate via the N-end rule pathway. It may be that *in vivo* 3D polymerase generated by HAV polyprotein processing is a classic N-end rule substrate in addition to being a substrate for the ubiquitin-conjugating pathway detected in this study. The rate at which the HAV 3D RNA polymerase is degraded may therefore be even more rapid in infected cells than in our *in vitro* systems.

A mutation in the E3 α -recognized signal in the 3C protease region did not reduce either the susceptibility of the HAV 3ABCD polyprotein or the 3CD diprotein toward ubiquitin-mediated degradation. These data, in addition to the fact that the 3ABC polyprotein proved to be a poor ubiquitin system substrate, seem to indicate the signal in the 3C protease is not readily recognized when the protease is linked with the adjacent P3 region proteins. In the case of the 3D polymerase-containing precursors the most straightforward interpretation of the data is that a signal in the 3D polymerase region is responsible for bringing about their ubiquitination and destruction. The 3D polymerase-containing precursors, such as the 3D polymerase alone and the HAV 3C protease, were found to be ubiquitinated initially at a single site, consistent with an initial ubiquitin conjugation event being catalyzed by a ubiquitin-protein ligase associated with the substrate at a single location. It must be considered, however, that the folded HAV polyprotein precursors may present E3 ligase-recognized protein destruction signals that are absent in either the mature 3C protease or the 3D polymerase.

As observed previously, the HAV 3C protease is not completely incorporated into conjugates with ubiquitin under the reaction conditions employed here (Gladding et al., 1997; Lawson et al., 1999), and the HAV 3D polymerase, 3ABCD polyprotein, and 3CD diprotein were also observed to be incompletely ubiquitinated. The incomplete ubiquitination of the 3C protease can be explained by the low k_{cat} value that characterizes the reaction and the slow dissociation of the E3 α -3C protease complexes (Lawson et al., 2001). A slow dissociation rate may also characterize complexes containing an E3 ligase and the HAV 3D polymerase or the 3D polymerase-containing precursors. The action of isopeptidases that catalyze ubiquitin removal may also contribute to the relatively low percentage of HAV substrates that were observed to be incorporated into ubiquitin-protein conjugates, but recent experiments using an isopeptidase inhibitor have demonstrated isopeptidase action alone cannot explain these results (P.E. Schlabach and T.G. Lawson, unpublished results). A comparison of the extent to which the HAV proteins and polyprotein precursors were incorporated into monoubiquitin conjugates suggests the HAV 3C protease undergoes the initial ubiquitin attachment event more readily than does the 3D polymerase or precursor polyproteins.

The discovery that both the HAV 3C protease and the 3D RNA polymerase are characterized by relatively short half-lives provides at least a partial explanation for why they are difficult to detect in HAV-infected cells. More research is clearly required to determine if the degradation of these proteins, or of the polyproteins that contain them, plays a role in mediating successful virus replication. The stable poliovirus 3C protease has been shown to produce cytopathic effects when overexpressed (Barco et al., 2000), and we have observed that purified HAV and EMCV 3C proteases are much more active than the poliovirus 3C protease in cleaving their respective polyprotein substrates *in trans* (T.G. Lawson et al., unpublished results). It is therefore possible that maintaining low concentrations of the HAV and EMCV 3C proteases in infected cells is needed to prevent premature cell death. It is more difficult to hypothesize why maintaining a low concentration of the RNA polymerase provides a benefit for HAV replication.

Materials and methods

Construction of plasmids and RNA transcripts

The preparation of the *in vitro* transcription plasmids pHAV3C, pHAV3C[LGV(32–34)AAA], and pHAV3C[LLV(39–41)AAA], carrying sequences coding for the nonmutated and mutated versions of the HAV 3C protease, and pP3C, carrying the poliovirus 3C protease-encoding sequence, has been previously described (Gladding et al., 1997; Lawson et al., 1999). The additional mutated HAV 3C protease-encoding plasmids pHAV3C[+A37], pHAV3C-

[L32A], pHAV3C[C172A], and pHAV3C[C172S] were constructed using pHAV3C as a template for PCR with the mutagenic primers 5'-CTTGGGAGTGAAAGATG-ATGGCTGGCTGCTTGTGCCTCC-3', 5'-GG-TTATGAATGCCGCGGGAGTGAAAGATGATTG-3', 5'-GGTCTTCTGGAATGGCTGGTGGGGCC-3', and 5'-CTTCTGGAATGTCGGGTGGGGCCTTGGTTTC-3', respectively, along with the flanking, vector DNA complementary primers 5'-CTCACATGTTCTTCTGCG-3' (coding strand), and 5'-AATACGACTCACTATAGGGC-3' (noncoding strand). The resulting DNA products were digested with *NcoI* and *EcoRI* and ligated into pGEM-3Z* (Lawson et al., 1994) at the same sites. pP3C-HAV3C-DS, carrying the sequence coding for the poliovirus 3C protease with an added N-terminal methionine and the amino acid sequence LGVKDDWLLV substituted at positions 28 through 37, was constructed using two rounds of PCR-based mutagenesis beginning with pP3C as a template. The sequence coding for AIL at positions 35 through 37 was first mutated to code for LLV using the mutagenic primer 5'-GGAGTCCACGACAACGTGCTTCTTGTAC-CAACCCACGCTTCACC-3' and the same flanking primers employed in the construction of the mutated HAV 3C protease-encoding plasmids described above. The resulting PCR product was digested with *NcoI* and *EcoRI* and ligated into pGEM-3Z* to produce pP3C[AIL(35–37)LLV]. The sequence coding for HDNV at position 31 through 34 was mutated to code for KDDW by using pP3C[AIL(35–37)LLV] as the template for PCR with the mutagenic primer 5'-CACTATGTTAGGAGTCAAGGACGACTGGC-TTCTTGTACCAACC-3' and the same flanking primers used above. The resulting PCR product was digested with *NcoI* and *EcoRI* and ligated into pGEM-3Z* to generate pP3C-HAV3C-DS.

pHAV3ABCD, carrying sequences coding for the HAV P3 (3ABCD) polyprotein with an added N-terminal methionine, and pHAV3CD, carrying sequences coding for the 3CD diprotein with an added N-terminal MESQ, were prepared as follows. The insert for pHAV3ABCD was amplified from pHAV/7 (Raychaudhuri et al., 1998), kindly provided by Dr. Suzanne Emerson, using the primers 5'-GGAGTTGTGGTCCATGGGAATTTTCAGATGATG-3' (coding strand) and 5'-GAAATTTTAAGAGAATTCGTT-TAAACAAATCATGAAAGGTCAC-3' (noncoding strand). The insert for pHAV3CD was amplified from pHAVT/7 using the same noncoding strand primer and the coding strand primer 5'-GATGCAGATCCCATGGAATCTCA-GTCAACTTTGG-3'. These DNA products were digested with *NcoI* and *EcoRI* and ligated into pGEM-3Z*. Versions of these constructs carrying a mutation coding for a LLV to AAA substitution at positions 39 through 41 in the 3C protease region (Lawson et al., 1999) were prepared as follows. pHAV3ABC(i) was constructed by ligating the 310-bp DNA *NcoI*-*HincII* fragment from pHAV3ABCD into pHAV3C[LLV(39–41)AAA] digested with the same enzymes. The 817-bp *NcoI*-*BbsI* fragment from

pHAV3ABC(i) was then ligated to the large fragment generated by digesting pHAV3ABCD with the same enzymes, to yield pHAV3ABC(i)D. To construct pHAV3C(i)D, the 3C(i)D coding region of pHAV3ABC(i)D was amplified by PCR using the coding strand primer 5'-GATGCAGATCCATGGAATCTCAGTCAACTTTGG-3' and the same noncoding strand vector sequence flanking primer employed in the construction of the mutated HAV 3C protease-encoding plasmids described earlier. This DNA product was digested with *NcoI* and *EcoRI* and ligated into pGEM-3Z*. pE5L'-HAV3ABC was constructed using a scheme similar to that previously reported (Jia et al., 1993). The 3ABC coding region was amplified from pHAV/7 by PCR using the primers 5'-CATGGAGTTGTGCGCGCAGGGAATTTCAGATGATGATAATGATAG-3' (coding strand) and 5'-GAACACTGAGTAATCTAGACTTTCATAATTCAC-TGACTTTCAATTTTC-3' (noncoding strand). The product was digested with *BssHII* and *XbaI* and ligated into the large DNA fragment generated by digesting pE5LVP0 (Parks et al., 1986) with the same enzymes. This resulted in a sequence coding for the 3ABC polyprotein with the added N-terminal amino acid sequence MATTMEQETCAQ and the added C-terminal amino acid sequence RIMKV placed downstream of the EMCV internal ribosome entry site (IRES).

Plasmids carrying sequences coding for the HAV, EMCV, and poliovirus 3D polymerases, pHAV3D, pE3D, and pP3D, respectively, were constructed as follows. The sequence coding for the HAV 3D polymerase with the added N-terminal amino acid sequence MESQ was amplified by PCR from pHAV3ABCD using the coding strand primer 5'-CAAAATATTGATAAGACCATGGAAAGTC-AGAGAATTATGAAAGTGGAG-3' and the same noncoding strand vector sequence flanking primer employed in constructions described above. The sequence coding for the EMCV 3D polymerase with an added methionine codon was amplified from pE3A'BCD (Oberst et al., 1993) using the same noncoding strand primer and the coding strand primer 5'-GTGAATGCCTTTGAGCCCATGGGTGCTCTCGAGAGATTG-3'. To eliminate the *NcoI* site present in the EMCV 3D DNA sequence, the CCC proline codon in this DNA product was changed to CCA by PCR-based mutagenesis using the mutagenic primer 5'-GAGGGGATGGACCCAATGGACAGGAACACC-3'. The sequence coding for the poliovirus 3D polymerase with the added N-terminal amino acid sequence MSQ was amplified from pT7-1 (Haller and Semler, 1992), kindly provided by Dr. Bert Semler, using the primers 5'-CTGAAGCGATC-ATACTTCACCATGGGTCAAGGTGAAATCCAGTGG-3' (coding strand) and 5'-GTCAATGACCCAATCGAATTC-GACTCAGGTAGGG-3' (noncoding strand). All of the final PCR products carrying the 3D polymerase coding sequences were digested with *NcoI* and *EcoRI* and ligated into pGEM-3Z*. The sequences of the insert regions in all of the plasmid constructs were confirmed by automated DNA sequence analysis.

For use in *in vitro* transcription reactions, all of the pGEM-3Z*-based plasmids were linearized by digestion with *EcoRI*. pHAV3D was also digested with *XhoI*. pE5L'-HAV3ABC was linearized by digestion with *XbaI*. *In vitro* RNA synthesis was carried out using SP6 RNA polymerase or, in the case of EL'-HAV 3ABC-encoding RNA programmed reactions, T7 RNA polymerase as previously described (Oberst et al., 1993; Lawson et al., 1994).

Assays for ubiquitin conjugation and proteasome-dependent degradation

³⁵S-labeled proteins and polyproteins were prepared by *in vitro* translation of the RNA transcripts described above in nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [³⁵S]methionine, as previously described (Oberst et al., 1993; Lawson et al., 1994). ³⁵S-labeled HAV 3D polymerase was partially purified from *in vitro* translation reaction mixtures by sedimentation through gradients of 5 to 20% sucrose (Oberst et al., 1993) followed by treatment with Q-Sepharose (Amersham Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.6, 1 mM DTT, and 0.1 mM EDTA (TDE buffer). Bound proteins were eluted from the resin using a step gradient of 0.1 to 0.5 M NaCl in the same buffer. The 3D polymerase eluted in 0.3 M NaCl. This fraction was desalted by dialysis against TDE buffer containing 5% (v/v) glycerol and concentrated to a volume equal to two times the original translation reaction mixture.

Viral proteins and polyproteins were evaluated as substrates for conjugation with ubiquitin in rabbit reticulocyte lysate using a procedure similar to that previously reported (Lawson et al., 1994). ³⁵S-labeled proteins and polyproteins were incubated at 30°C in reaction mixtures containing 20 mM HEPES-KOH, pH 7.5, 100 mM KC₂H₄O₂, 1 mM Mg(C₂H₄O₂)₂, 1 mM DTT, and 0.1 mg/ml cycloheximide (Sigma) in 50% (v/v) nuclease-treated reticulocyte lysate containing an energy-generating system in the absence or in the presence of either 0.1 mM methylated ubiquitin (prepared as described; Hershko and Heller, 1985; Lawson et al., 1994) or 0.025 mM GST-ubiquitin (Boston Biochemical). Aliquots were removed from the mixtures at the indicated times, precipitated with 5 volumes of acetone, and analyzed by SDS-PAGE and autoradiography. In some cases 0.025 mM GST-ubiquitin was included in the translation reaction mixtures used to prepare the labeled proteins, and aliquots from these mixtures were removed and analyzed in the same manner. Partially purified ³⁵S-labeled HAV 3D RNA polymerase was also evaluated as a substrate for ubiquitination in HeLa cell S-100 extract (Boston Biochemical) by incubating 10 μl of labeled protein preparation in 25 μl reaction mixtures containing 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 4 mM ATP, 1 mM DTT, 10 mM creatine phosphate, 0.6 unit/ml creatine phosphokinase, 0.6 unit/ml pyrophosphatase, 0.25 μM ubiquitin aldehyde (Boston Biochemical), and 0.82 mg/ml S-100 protein, in the absence or in the presence of 0.1 mM methylated ubiquitin or 0.025

mM GST-ubiquitin, at 30°C. Aliquots were removed from the mixtures at the indicated times, precipitated with 5 volumes of acetone, and analyzed by SDS-PAGE and autoradiography. The incorporation of methylated ubiquitin and GST-ubiquitin into conjugates with the labeled proteins was determined by liquid scintillation counting of gel slices containing the unconjugated protein and protein conjugated with the ubiquitin derivative (Oberst et al., 1993; Lawson et al., 1994). The incorporation was expressed as the percentage of the total cpm (in the unconjugated plus conjugated proteins) present in the conjugated products alone.

The stability of the viral proteins and polyproteins in reticulocyte lysate was evaluated using methods similar to those previously employed (Oberst et al., 1993; Lawson et al., 1994). ³⁵S-labeled proteins and polyproteins were incubated at 30°C in reaction mixtures containing 20 mM HEPES-KOH, pH 7.5, 100 mM KC₂H₄O₂, 1 mM Mg(C₂H₄O₂)₂, 1 mM DTT, 0.1 mg/ml cycloheximide, and 0.1 mM ubiquitin (Sigma) in 50% (v/v) nuclease-treated reticulocyte lysate containing an energy generating system. Aliquots were removed from the mixtures at the indicated times and analyzed by SDS-PAGE and autoradiography. In some cases, the quantity of individual labeled proteins present in the gels was determined using liquid scintillation counting. To test for the involvement of proteasome activity in protein disappearance, some reactions were carried out in the presence of added 4 mM adenosine 5'-O-(3-thiophosphate) (ATP-γ-S; Sigma) plus 4 mM additional Mg(C₂H₄O₂)₂.

Acknowledgments

This work was supported by National Science Foundation RUI Awards MCB-9974497 and MCB-0210188 and a Henry Dreyfus Teacher Scholar Award.

References

- Allaire, M., Chernaia, M.M., Malcolm, B.A., James, M.N.G., 1994. Picornaviral 3C cysteine proteinases have a fold similar to chymotrypsin-like serine proteinases. *Nature* 369, 72–76.
- Anderson, D.A., Ross, B.C., Locarnini, S.A., 1988. Restricted replication of hepatitis A virus in cell culture: encapsidation of viral RNA depletes the pool of RNA available for replication. *J. Virol.* 62, 4201–4206.
- Barco, A., Feduchi, E., Carrasco, L., 2000. Poliovirus protease 3C(pro) kills cells by apoptosis. *Virology* 266, 352–360.
- Bergmann, E.M., Mosimann, S.C., Chernaia, M.M., Malcolm, B.A., James, M.N.G., 1997. The refined crystal structure of the 3C gene product from hepatitis A virus: specific proteinase activity and RNA recognition. *J. Virol.* 71, 2436–2448.
- Charini, W.A., Burns, C.C., Ehrenfeld, E., Semler, B.L., 1991. trans rescue of a mutant poliovirus RNA polymerase function. *J. Virol.* 65, 2655–2665.
- Ciechanover, A., Orian, A., Schwartz, A.L., 2000. Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 22, 442–451.

- Ciechanover, A., Shkedy, D., Oren, M., Bercovich, B., 1994. Degradation of the tumor suppressor protein p53 by the ubiquitin-mediated proteolytic system requires a novel species of ubiquitin-carrier protein, E2. *J. Biol. Chem.* 269, 9582–9589.
- Emerson, S.U., Huang, Y.K., Purcell, R.H., 1993. 2B and 2C mutations are essential but mutations throughout the genome of HAV contribute to adaptation to cell culture. *Virology* 194, 475–480.
- Funkhouser, A.W., Schultz, D.E., Lemon, S.M., Purcell, R.H., Emerson, S.U., 1999. Hepatitis A virus translation is rate-limiting for virus replication in MRC-5 cells. *Virology* 254, 268–278.
- Gladding, R.L., Haas, A.L., Gronros, D.L., Lawson, T.G., 1997. Evaluation of the susceptibility of the 3C proteases of hepatitis A virus and poliovirus to degradation by the ubiquitin-mediated proteolytic system. *Biochem. Biophys. Res. Commun.* 238, 119–125.
- Graff, J., Ehrenfeld, E., 1998. Coding sequences enhance internal initiation of translation by hepatitis A virus RNA in vitro. *J. Virol.* 72, 3571–3577.
- Haller, A.A., Semler, B.L., 1992. Linker scanning mutagenesis of the internal ribosome entry site of poliovirus RNA. *J. Virol.* 66, 5075–5086.
- Hershko, A., Ciechanover, A., 1998. The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.
- Hershko, A., Heller, H., 1985. Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. *Biochem. Biophys. Res. Commun.* 128, 1079–1086.
- Jen, G., Thach, R.E., 1982. Inhibition of host translation in encephalomyocarditis virus-infected L cells: a novel mechanism. *J. Virol.* 43, 250–261.
- Jia, X.-Y., Ehrenfeld, E., Summers, D.F., 1991a. Proteolytic activity of hepatitis A virus 3C protein. *J. Virol.* 65, 2595–2600.
- Jia, X.-Y., Scheper, G., Brown, D., Updike, W., Harmon, S., Richards, O., Summers, D., Ehrenfeld, E., 1991b. Translation of hepatitis A virus RNA in vitro: aberrant internal initiations influenced by 5' noncoding region. *Virology* 182, 712–722.
- Jia, X.-Y., Summers, D.F., Ehrenfeld, E., 1993. Primary cleavage of the HAV capsid protein precursor in the middle of the proposed 2A coding region. *Virology* 193, 515–519.
- Jurgensen, D., Kusov, Y.Y., Facke, M., Krausslich, H.G., Gauss-Muller, V., 1993. Cell-free translation and proteolytic processing of the hepatitis A virus polyprotein. *J. Gen. Virol.* 74, 677–683.
- Lam, Y.A., Lawson, T.G., Velayutham, M., Zweier, J.L., Pickart, C.M., 2002. A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* 416, 763–767.
- Laney, J., Hochstrasser, M., 1999. Substrate targeting in the ubiquitin system. *Cell* 97, 427–430.
- Lawson, T.G., Gronros, D.L., Evans, P.E., Bastien, M.C., Michalewich, K.M., Clark, J.K., Edmonds, J.H., Graber, K.H., Werner, J.A., Lurvey, B.A., Cate, J.M., 1999. Identification and characterization of a protein destruction signal in the encephalomyocarditis virus 3C protease. *J. Biol. Chem.* 274, 9871–9980.
- Lawson, T.G., Gronros, D.L., Werner, J.A., Wey, A.C., DiGeorge, A.M., Lockhart, J.L., Wilson, J.W., Wintrobe, P.L., 1994. The encephalomyocarditis virus 3C protease is a substrate for the ubiquitin-mediated proteolytic system. *J. Biol. Chem.* 269, 28429–28435.
- Lawson, T.G., Smith, L.L., Palmenberg, A.C., Thach, R.E., 1989. Inducible expression of encephalomyocarditis virus 3C protease activity in stably transformed mouse cell lines. *J. Virol.* 63, 5013–5022.
- Lawson, T.G., Sweep, M.E., Schlax, P.E., Bohnsack, R.N., Haas, A.L., 2001. Kinetic analysis of the conjugation of ubiquitin to picornavirus 3C proteases catalyzed by the mammalian ubiquitin-protein ligase E3 α . *J. Biol. Chem.* 276, 39629–39637.
- Oberst, M.D., Gollan, T.J., Gupta, M., Peura, S.R., Zydlowski, J.D., Sudarsanan, P., Lawson, T.G., 1993. The encephalomyocarditis virus 3C protease is rapidly degraded by an ATP-dependent proteolytic system in reticulocyte lysate. *Virology* 193, 28–40.
- Parks, G.D., Duke, G.M., Palmenberg, A.C., 1986. Encephalomyocarditis virus 3C protease: efficient cell-free expression from clones which link viral 5' noncoding sequences to the P3 region. *J. Virol.* 60, 376–384.
- Pickart, C.M., 2000. Ubiquitin in chains. *Trends Biochem. Sci.* 25, 544–548.
- Pickart, C.M., 2001. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70, 505–533.
- Probst, C., Jecht, M., Gauss-Muller, V., 1998. Processing of proteinase precursors and their effect on hepatitis A virus particle formation. *J. Virol.* 72, 8013–8020.
- Raychaudhuri, G., Govindarajan, S., Shapiro, M., Purcell, R.H., Emerson, S.U., 1998. Utilization of chimeras between human (HM-175) and simian (AGM-27) strains of hepatitis A virus to study the molecular basis of virulence. *J. Virol.* 72, 7467–7475.
- Reiss, Y., Kaim, D., Hershko, A., 1988. Specificity of binding of NH₂-terminal residue of proteins to ubiquitin-protein ligase. Use of amino acid derivatives to characterize specific binding sites. *J. Biol. Chem.* 263, 2693–2698.
- Scheffner, M., Huibregtse, J.M., Vierstra, R.D., Howley, P.M., 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75, 495–505.
- Schultheiss, T., Kusov, Y.Y., Gauss-Muller, V., 1994. Proteinase 3C of hepatitis A virus cleaves the HAV polyprotein P2-P3 at all sites including VP1/2A and 2A/2B. *Virology* 198, 275–281.
- Schultz, D.E., Honda, M., Whetter, L.E., McKnight, K.L., Lemon, S.M., 1996. Mutations within the 5' nontranslated RNA of cell culture-adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey kidney cells. *J. Virol.* 70, 1041–1049.
- Tesar, M., Pak, I., Jia, X.-Y., Richards, O.C., Summers, D.F., Ehrenfeld, E., 1994. Expression of hepatitis A virus precursor protein P3 in vivo and in vitro: polyprotein processing of the 3CD cleavage site. *Virology* 198, 524–533.
- Ticehurst, J.R., Cohen, J.L., Feinstone, S.M., Purcell, R.H., Jansen, R.W., Lemon, S.M., 1989. Replication of hepatitis A virus: new ideas from studies with cloned cDNA, in: Ehrenfeld, E., Semler, B.L. (Eds.), *Molecular Aspects of Picornavirus Infection and Detection*. American Society for Microbiology, Washington, DC, pp. 27–50.
- Updike, W.S., Tesar, M., Ehrenfeld, E., 1991. Detection of hepatitis A virus proteins in infected BS-C-1 cells. *Virology* 185, 411–418.
- Varshavsky, A., 1996. The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* 93, 12142–12149.
- Varshavsky, A., 1997. The ubiquitin system. *Trends Biochem. Sci.* 22, 383–387.
- Voges, D., Zwickl, P., Baumeister, W., 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* 68, 1015–1068.
- Whetter, L.E., Day, S.P., Elroy-Stein, O., Brown, E.A., Lemon, S.M., 1994. Low efficiency of the 5' nontranslated region of hepatitis A virus RNA in directing cap-independent translation in permissive monkey kidney cells. *J. Virol.* 68, 5253–5263.
- Yi, M., Lemon, S.M., 2002. Replication of subgenomic hepatitis A virus RNAs expressing firefly luciferase is enhanced by mutations associated with adaptation of virus to growth in cultured cells. *J. Virol.* 76, 1171–1180.
- Yi, M., Schultz, D.E., Lemon, S.M., 2000. Functional significance of the interaction of hepatitis A virus RNA with glyceraldehyde 3-phosphate dehydrogenase (GAPDH): opposing effects of GAPDH and polypyrimidine tract binding protein on internal ribosome entry site function. *J. Virol.* 74, 6459–6488.
- Zhang, H., Chao, S.F., Ping, L.H., Grace, K., Clarke, B., Lemon, S.M., 1995. An infectious cDNA clone of a cytopathic hepatitis A virus: genomic regions associated with rapid replication and cytopathic effect. *Virology* 212, 686–697.